Expression of transcription factors and matrix genes in response to serum stimulus in vascular smooth muscle cells

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During atherogenesis vascular smooth muscle cells are converted from a contractile into a synthetic phenotype characterized by enhanced matrix production. The transcription factors Gax and GATA-6 are considered negative, and Oct-1 positive regulators of the synthetic phenotype. Since the phenotype transition can be induced by culturing the cells with serum, we followed the expression of Gax, GATA-6 and Oct-1, integrins and matrix genes in quiescent porcine vascular smooth muscle cells after serum application. Comparisons were made between enzymatically released primary smooth muscle cells and cells grown out from explants of the medial layer of porcine aorta. The serum-mediated down-regulation of Gax was more intense than that of GATA-6, and stronger in explant-derived than in primary cells. Serum was without influence on the expression of Oct-1. Changes in the expression of the transcription factors preceded the induction of integrin α2 and the down-regulation of decorin, while mRNAs for laminin β1 and osteopontin rose immediately after serum stimulation. Primary cells reacted more rapidly than explant cells with respect to changes in laminin isoforms. Studies with a Gax-expressing adenovirus indicated that among all the gene products tested only the expression of integrin α2 responded to Gax induction. Thus, our data show that *i*) Gax should be considered a transcription factor being directly responsible for only few aspects of the phenotypic conversion of smooth muscle cells and that *ii*) explant cells may represent a subpopulation of smooth muscle cells, which differ from the total population of smooth muscle cells, as obtained in primary culture, in their response to serum stimuli.

Abbreviations. Adv Adenovirus serotype 5. – CDS Coding sequence. – FBS Foetal bovine serum. – PCR Polymerase chain reaction. – RT Reverse transcription. – VSMC Vascular smooth muscle cells

Introduction

One of the hallmarks during the development of atherosclerotic lesions is the reversible shift of vascular smooth muscle cells (VSMC) from a differentiated, contractile phenotype to a less differentiated synthetic phenotype. This transition occurs as a response to vascular injury (Ross, 1993) and is accompanied by reorganization of the cytoskeleton, increased biosynthesis of extracellular matrix components and by VSMC proliferation and migration (Campbell et al., 1988, 1991, Dzau et al., 2002). Sequential, and presumably coordinated waves of changes in the expression of dozens of genes in response to injury have recently been described (Tai et al., 2000). However, the mechanisms underlying these changes in morphology and function of VSMC are still incompletely understood as are the factors transmitting intracellularly the signals provided from the extracellular environment through tensile forces, growth factors, extracellular matrix molecules, and other components.

Several transcription factors have been considered to be central players in the phenotypic transition of VSMC. Special attention has been paid to Gax, a member of the homeobox genes, since the expression of Gax is largely confined to the cardiovascular system in the adult. Gax is rapidly down-regulated when quiescent VSMC enter the cell cycle (Gorski et al., 1993; LePage et al., 1994). Another transcription factor

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displaying similar kinetics during G_o/G_1 transition is GATA-6 (Suzuki et al., 1996), which is the only member of the GATA family expressed in the adult vasculature (Laverriere et al., 1994; Jiang et al., 1998; Perlman et al., 1998). The down-regulation of Gax and GATA-6 seems to be essential for entry into the S-phase because adenoviral gene transfer of these factors leads to p21-dependent cell cycle arrest and reduced neointimal hyperplasia and luminal stenosis (Perlman et al., 1998; Smith et al., 1997; Maillard et al., 1997; Mano et al., 1999). An opposite role has been ascribed to Oct-1 (Sturm et al., 1988), which is undetectable in VSMC in situ, but is up-regulated in proliferating cultured cells (Lakin et al., 1995; Weiser et al., 1997).

It is likely, although unconfirmed, that VSMC respond non-uniformly towards changes in the expression of these transcription factors, because VSMC of intimal atherosclerotic lesions are of clonal origin, and small cell clones may also be present in the normal intima (Murry et al., 1997). The explanation for this clonal expansion is not yet known, but these findings illustrate the need for using well-defined models in studies on phenotype transition.

The transition from a contractile into a synthetic phenotype can be elicited in vitro, at least in part, by maintaining cultures of VSMC in the quiescent state and then stimulating them with PDGF-BB (Gorski and Walsh, 1995) or whole FBS (Thyberg, 1996). Upon serum treatment, cells enter the G_1 phase and eventually acquire the synthetic phenotype. In the present study we investigated the short-term consequences of serum stimulation of porcine VSMC on the expression of the transcription factors Gax, GATA-6 and Oct-1, and on selected integrins and extracellular matrix components. The integrin chains α1 and α2 were chosen because α1β1 and α2β1 integrins may be reciprocally expressed during VSMC dedifferentiation and may play a role in cell migration on type I collagen (Skinner et al., 1994). The integrin α2 chain is also of major importance for platelet adhesion on collagen (Holtkötter et al., 2002). Consequently, the α1 (I) collagen chain and the type-I collagen-associated proteoglycans decorin and biglycan were studied which are characteristic constituents of fibrous tissue (Riessen et al., 1994; Yamakawa et al., 2000). Biglycan seems additionally to be required for endothelial cell migration (Kinsella et al., 1997). As VSMC are encapsulated by a basement membrane, selected laminin chains (Tunggal et al., 2000) were also investigated. The laminin β2 chain has been found in adult human media, whereas the β1 chain which represents the dominating β chain in embryonic vessels, was re-expressed during intimal thickening (Glukhova et al., 1993). Furthermore, the laminin α4 chain, which is a component of the laminins 8 (α4β1γ1) and 9 (α4β2γ1), is expressed in normal smooth muscle cells (Kortesmaa et al., 2000) and also present in atherosclerotic plaques while in case of laminins 10 (α5β1γ1) and 11 (α5β2α1) the former one is typically found in lesions (J. Rauterberg, unpublished result). Hence, our studies included the quantification of the expression of several relevant laminin genes. Additionally, osteopontin was investigated, because it was used as a marker of arterial injury (Giachelli et al., 1995).

To analyse the serum-mediated changes in gene expression, primary VSMC were used, which were obtained either from an elastase/collagenase digest of the whole aortic media or as cells that had grown out from explant cultures of this tissue. While enzymatically released cells are considered to represent the whole spectrum of aortic VSMC, cells grown out from explanted tissue represent a population with high migratory activity during their culture history. Our findings indicate that the two populations of cells react differently to serum stimulation.

Materials and methods

RNA isolation and reverse transcription

Total RNA and mRNA from porcine VSMC and from the freshly prepared medial layer of thoracic porcine aorta were isolated using Qiagen RNA isolation kits (Qiagen, Hilden, Germany) and digested with RNase-free DNase. RT was performed with Omniscript Reverse Transcriptase (Qiagen) according to the instructions of the manufacturer.

PCR-based cloning

Reverse transcriptase PCR (RT-PCR) was used to establish porcine sequences of the following cDNAs: Gax, GATA-6, collagen α1 (I) chain, integrin chains α1 and α2, laminin chains α4, β1, β2, and γ1. Full-length coding sequences were established for porcine Gax and GATA-6. PCR primer pairs were deduced from sequences highly conserved between humans, mice and rats.

Gax (915 bp, CDS: bp 4 – 915) was cloned by using GCYATGGAA-CACCCSCTC as forward and TCAKARGTGKGCGTGCTC as reverse and complement primer. The cycle parameters were 30 cycles at 94 °C, 63 °C, and 72 °C for 1 min each. GATA-6 cloning required a two-step procedure. First, the 5′-end (bp – 15 to 940) was amplified by employing GAGCAGCCGGAGGARATG and GTTCACCCTC-GGCGTTTCTGC as primer pair and PCR conditions as above, except of an annealing temperature of 60 °C. Then, the 3′-terminal part (bp 873 to 2643) was amplified by using a cDNA as template which had been synthesized as described above except that the RNA was primed with the 3′-RACE primer TCGGACAGTCGACATCGGTAA$(T)_{17}$. The first cycle with GTGCGTGAACTGCGGCTCCATC and TCGGA-CAGTCGACATCGGTAA was 95 °C for 15 min, 60 °C for 2 min and 72 °C for 40 min followed by 30 cycles at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 3 min. The 3′-RACE PCR product was used as template for nested PCR with the primers TGGATTGTCCTGTGCCAACT and TCGGACAGTCGACATCGGTAA under otherwise identical conditions. The two partly overlapping fragments contain the whole CDS of GATA-6 (bp 1 – 1341).

cDNAs for matrix proteins and integrins did not comprise the full-length coding sequence as they were used only to develop appropriate real-time RT-PCR conditions. The primers used and the reaction conditions are listed in Table I. The PCR products were obtained after agarose gel electrophoresis, subcloned into pCRII-TOPO (Invitrogen, Karlsruhe, Germany) and sequenced.

Northern blotting

Northern blottings were performed as described previously (Schaefer et al., 2002). The probe for Gax was the whole coding sequence, and for GATA-6 a 221-bp fragment. For hybridization with the GATA-6 probe 30 μg of total RNA were required, while for hybridization with the Gax probe 4 μg of mRNA were needed. Probes were labelled with [^{32}P]dCTP by using Ready-To-Go DNA Labelling Beads (Amersham Pharmacia Biotech, Freiburg, Germany) according to the instructions of the manufacturer. Hybridizations were performed in QickHyb solution (Stratagene, Amsterdam, The Netherlands) at 60 °C (Gax and Oct-1) and 68 °C (GATA-6), respectively.

Real-time PCR

Semiquantitative real-time PCR was performed using the GeneAmp 5700 Sequence Detection System and SYBR Green PCR Core Reagents (PE Applied Biosystems, Weiterstadt, Germany). Cycling conditions were 50 °C for 2 min (uracil N-glycosylase incubation), 95 °C for 10 min (denaturation and AmpliTaq Gold activation), 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Primer sequences were derived from the porcine

Tab. I. Primer sequences for PCR cloning and real-time PCR.

Gene	PCR cloning	Real-time PCR
Collagen α1(I)[1]	TGGTGACAAGGGTGAGACAG TTCCAGTCAGAGTGGCACAT	TTCCAGTCAGAGTGGCACAT AGCCAGCAGATCGAGAACAT
Integrin α1[2]	CATCAGGTGGGGATGGTAAG CCCATTTTCTGGATCGGTAA	TCTCACAGAGTCCCGAAAGTC CAAAGAAACATCGCAGTTCG
Integrin α2[3]	GGATCCGATGGAGCCTTTAGGA TAGGCTTCAAGGGCAGGGCTA	CTAGTGCCAGGGTTTTCCAG TTGTTCTGAGCACATCATCCA
Laminin α4[4]	TCTCAGCAATCTCCAGCTCA CCAAGCGTGGTGTCAGTAGA	TTCACTATGACCTGCCCATTT TTTGAAGTCCGTCCCAGAAG
Laminin β1[5]	GGGTGCCTTGGATAGCATTA TGGAGAGCTGTTCCACTTCA	AGGCAGAGGAGAGGGTGAAT GGGACTCTTGCTCCAACATC
Laminin β2[6]	CTGGACAAGGCTAATGCTTC ACCTGCCATCCTCTCCTGTA	CTAATGCTTCCAGGGGACAG GGCCACCATCTCAATGCTAT
Laminin γ1[7]	GCCCAGGATGTCAAAGATGT ATGTACTCGGGCAGCTTGTT	TTTAATGGATCGCCTCCAGA CAAGTTTCCGGTCTCCTCAA
Decorin		AAAGTGCGAAAGGCTGTGTT GAAAGCTCCGTTTTCAATGC
Biglycan		ACAGTGGCTTTGAACCTGGA TCATCCTGATCTGGTTGTGG
Osteopontin		TGTGTGGCTCTGGAAACAAA CGTCGACTAAACCCTGAAGC
GAPDH		CTCCCTCAAGATCGTCAGCAA CAGTCTTCTGGGTGGCAGTGA

[1] 33 cycles; 1 min 94 °C, 1 min 58.5 °C, 1 min 72 °C; 3 mM $MgCl_2$
[2] 30 cycles; 1 min 94 °C, 1 min 55 °C, 1 min 72 °C; 5 mM $MgCl_2$
[3] 32 cycles; 1 min 94 °C, 1 min 59 °C, 45 s 72 °C; 3.5 mM $MgCl_2$
[4] 30 cycles; 1 min 94 °C, 1 min 56 °C, 1 min 72 °C; 2.25 mM $MgCl_2$
[5] 30 cycles; 45 s 94 °C, 45 s 60 °C, 45 s 72 °C; 3 mM $MgCl_2$
[6] 32 cycles; 45 s 94 °C, 45 s 54 °C, 45 s 72 °C; 3 mM $MgCl_2$
[7] 30 cycles; 1 min 94 °C, 1 min 55 °C, 1 min 72 °C; 5 mM $MgCl_2$

cDNA sequences described above and are listed in Table I. The primer pairs for the transcription factors were AAGCAGAATTTGCCCATCAT and TTGGAGCCAGACTTTCACCT for Gax, CAAGTGCCTACTCGCCCTAC and GCGCGACTCTGTAGACTGTG for GATA-6, and GAGGAGCCCAGTGACCTTGA and CCCATAGCAAGCCCAACATC for Oct-1. The specifity of the PCR products was verified by dissociation curve analysis, agarose gel electrophoresis, and subcloning and sequencing of the products. Quantification was carried out as described (Winer et al., 1999). As a control, the PCR reactions were also performed without reverse transcription.

Construction of Adv-Gax

Porcine Gax cDNA was excised from the cloning vector pCRII-TOPO with KpnI and Eco RI and ligated into the adenovirus shuttle vector pAC-EF1. This vector is a derivative of pACCMV (Gluzman et al., 1982) and harbours the Adv sequences between 0.0 and 1.3 and between 9.3 and 17.0 map units. The region between map units 1.3 and 9.3 is replaced by the EF-1 promoter, the multicloning site and a BGH polyadenylation signal. Upon amplification of the vector, the integrity and orientation of the insert was verified by DNA sequence analysis. Plasmid DNA was purified by CsCl density gradient centrifugation and used to co-transfect 293 HEK cells (Becker et al., 1994) with the pJM17 plasmid, which contains the adenovirus type 5 genome but lacks the E1 region and has a non-functional E3 gene (McGrory et al., 1988). When recombination between the two plasmids had occurred, the virus was subsequently plaque purified, grown in large quantity, and purified by CsCl gradient centrifugation. The titre of the virus, determined by limiting dilution plaque assay, was 2×10^8 plaque-forming units (pfu)/ml. In addition, the number of virus copies was estimated with adenovirus-specific primers (sense: 5′-GTAGAGTCATAATCGGTCATCAGG-3′, antisense: 5′-TTTATATGGTACCGGGAGGTGGTG-3′). An insert-free adenovirus, Adv dl312, a kind gift of Dr. Brian C. O'Connell, University of Dublin, Ireland, was used for control purposes.

Cell culture

VSMC were isolated from porcine aortic tissue either by the explant method (Vischer and Buddecke, 1985) or by enzymatic digestion as described (Thie et al., 1991). Media pieces from freshly slaughtered animals were maintained in DMEM, supplemented with 10% FBS, 2 mM glutamine and antibiotics. The medium was replaced at 4-day intervals, and outgrowth of cells occurred within 7–10 days in 95% of the explants. These explant cells were used for experiments at passages two to four.

To obtain primary VSMC, media pieces were incubated with collagenase (Worthington, Lakewood, NJ, USA; 1 mg/ml, 142 units/mg) and elastase (type III, Sigma, Munich, Germany; 0.5 mg/ml, 3.8 units/mg) in serum-free DMEM at 37 °C for 1 h. Thereafter, the solution was replaced by an enzyme solution containing only collagenase (1 mg/ml), and the media pieces were incubated until all of the tissue was dispersed. The cells were recovered by centrifugation and incubated with DMEM containing 4 mM glutamine, antibiotics and 10% FBS to enable cell adherence. On the next day the cells were placed in MCDB-131 medium (Sigma) supplemented with ITS (insulin, transferrin, selenite) premix (Becton Dickinson, Heidelberg, Germany). This medium supports the maintenance of the contractile phenotype during several weeks in culture, as shown by the expression of myosin isoforms and ultrastructural analysis (F. Echtermeyer and J. Rauterberg, unpublished results). Serum stimulation was performed after 5 to 8 days of culture under serum-free conditions.

Immunohistochemistry

Indirect immunofluorescence analysis of cultured cells was performed by allowing primary and explant cells to attach to glass coverslips overnight in the presence of 10% FBS in MCDB-131 medium. Cells were cultured for 5 days in serum-free medium and stained for the heavy myosin isoforms SM1, SM2 and SMemb (Yamasa, Tokyo, Japan) and for smoothelin (Chemicon, Hofheim, Germany). Counterstaining was with DAPI (Vector, Burlingame, CA, USA). Cryostat sections of human coronary heart specimens were obtained from explanted hearts of patients undergoing cardiac transplantation, and further treated as described (Weitkamp et al., 1999). For double staining of integrin α chains, a rabbit polyclonal antibody against α1 integrin and a monoclonal mouse antibody (clone P1E6) against the α2 chain (Chemicon) were used. Laminin β1, β2 and γ1 chains were visualized by employing a rat monoclonal anti-β1 antibody (Chemicon), a mouse monoclonal anti-β2 antibody (clone C4, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, USA), and a mouse monoclonal antibody (clone D18, Developmental Studies Hybridoma Bank) against γ1, respectively. Secondary antibodies were either Cy2- or Cy3-conjugated AffinityPure $F(ab)_2$ fragment goat anti-rabbit IgG and anti-mouse IgG (Dianova, Hamburg, Germany). Nuclei were stained with Hoechst 33258 dye, and the sections were embedded in fluorescence mounting medium (Dako, Hamburg, Germany).

In-situ hybridization

For in-situ hybridization experiments Gax cDNA was cloned into the pGEM-7Zf(+) vector (Promega) to obtain the respective digoxigenin-labelled sense and antisense probes according to the protocol provided by the manufacturer. Cryostat sections of porcine and human tissues were fixed as described (Schaefer et al., 2001) and digested with proteinase K (5 μg/ml) in 50 mM Tris/HCl buffer, pH 8.0, containing 5 mM EDTA for 20 min at 37 °C. After further fixation with 4% paraformaldehyde for 10 min at ambient temperature and extensive washing with 18 mM sodium phosphate, pH 7.4/0.15 M NaCl, the sections were further treated with 0.1 M triethanolamine/25 mM acetic anhydride for 10 min and then dehydrated in an ascending ethanol series. Hybridization was performed at 55 °C for porcine and at 53 °C for human samples, respectively, under conditions exactly as described (Schaefer et al., 2001).

Viral infection of VSMC

VSCM ($\sim 1.5 \times 10^6$ cells/7-cm^2 dish) were cultured for 24 h with 2 ml/dish of MCDB-131 medium supplemented as described above. The medium was then replaced by 500 μl/dish of MEM containing 2% heat-inactivated FBS, 2 mM glutamine, antibiotics, and up to 200 pfu/cell of adenovirus. After 90 min of incubation, 1.5 ml/dish of MCDB-131 medium were added, and incubation continued for further 24 h. Subsequently, medium was removed, and the cells were either challenged with 10% FBS or maintained without stimulation as described above. At the times indicated, cells were harvested for total RNA preparation and real-time RT-PCR.

Bacterial expression of Gax for raising a polyclonal antiserum

Gax cDNA was cloned into the prokaryotic expression vector pET28(+) (Novagen, Schwalbach, Germany) in frame with an upstream located His-tag sequence and used to transfect *E. coli* BL21(DE3)pLysS (Novagen). After induction of protein expression Gax was purified on a Ni-NTA-agarose (Qiagen) column according to the manufacturer's recommendation. For raising a polyclonal antiserum against porcine Gax, a rabbit was intracutaneously injected on days 0, 20 and 32 with 100 μg of Gax protein/injection which had been suspended in complete (day 0) or incomplete (days 20 and 32) Freund's adjuvant. Serum was prepared from blood drawn on day 39.

Results

Molecular cloning and primary structure of Gax and GATA-6

VSMC from porcine aorta are easily obtainable for studies on their phenotype transition and were, therefore, used in the present investigation. Consequently, porcine sequences of Gax and GATA-6 had first to be elucidated, whereas the coding sequence of porcine Oct-1 was already available (GenBank/EMBL Accession No. L38524). The full-length coding sequences of porcine Gax (GenBank/EMBL Accession No. AF295686) and GATA-6 (GenBank/EMBL Accession No. AF295687) were obtained as described in Materials and methods. The sequence of Gax shows 92% and 89% homology, respectively, with the human (LePage et al., 1994) and the rat (Gorski et al., 1993) sequence. The amino acid sequences of the porcine and human protein differ in only six amino acids, two of which are part of the homeodomain. Both, the porcine and the rat proteins contain a stretch of 12 consecutive histidine residues whereas the human protein contains 11.

Similarly, porcine GATA-6 cDNA is highly homologous with its human (88%; Suzuki et al., 1996), rat (83%; Tamura et al., 1993), mouse (83%; Morrisey et al., 1996), chicken (81%; Laverriere et al., 1994) and frog (79%; Gove et al., 1997) counterparts. The 3′-untranslated regions of the porcine and human sequences show a comparably high degree of similarity. The amino acid sequence of the porcine and the human transcription factor differ by 25 amino acid substitutions, one insertion and a deletion of three sequential histidine residues. Like the human and rat protein, porcine GATA-6 contains stretches of alanine (amino acids 27 – 37) and histidine (amino acids 178 – 184) residues, which are lacking in chicken and frog.

Expression of porcine Gax, GATA-6 and Oct-1

In order to verify the presence of mRNAs of these transcription factors in VSMC, Northern blot analyses of aortic media and of early-passage cells were performed. Transcripts of 2.6 kb for Gax and of 3.9 kb for GATA-6 were found (data not shown). Values of 2.3 kb (Gorski et al., 1993) and 3.2 kb (Shaw-White et al., 1999) were given for the respective rodent mRNA species. Oct-1 mRNA could be detected only by RT-PCR which is in accordance with the low abundancy of this transcription factor even in serum-stimulated VSMC (Weiser et al., 1997).

Different kinetics of serum-dependent down-regulation of Gax and GATA-6

In light of the hypothesis that VSMC do not represent a homogeneous population, the transcriptional regulation of primary and explant cells was compared. Primary cells were maintained in a quiescent state in MCDB-131 medium, which supports the contractile phenotype. Immunohistochemical data indicated the re-conversion of explant-derived cells into a contractile and quiescent state by maintaining them for at least 5 days in MCDB-131 medium (Fig. 1). While the SM2 form of smooth muscle myosin was nearly absent in cells cultured in the presence of serum, this isoform was clearly detectable in serum-free cultures, especially at high cell density. Primary cells, obtained from the collagenase/elastase digest of the medial layer of the aorta, expressed at the most traces of SM2 unless they were kept with serum (result not shown). The presence of the isoforms SM1 and SMemb (not shown), on the

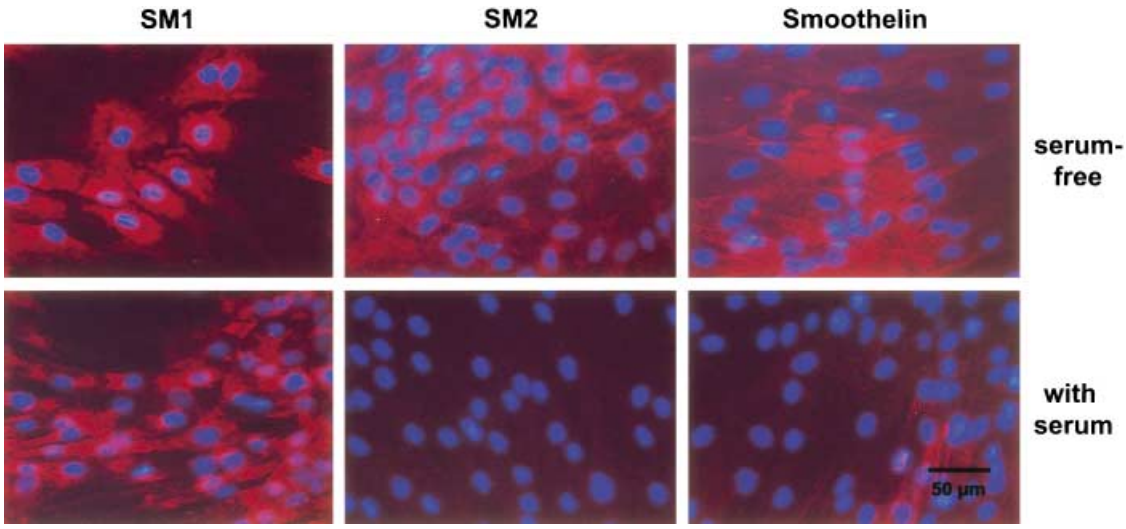

Fig. 1. Immunohistochemical staining of explant-derived VSMC for heavy chain myosin isoforms SM1 and SM2 and for smoothelin. Cultures were maintained in MCDB-131 medium, including insulin, transferrin and selenite, either without or with 10% FBS for 5 days prior to analysis.

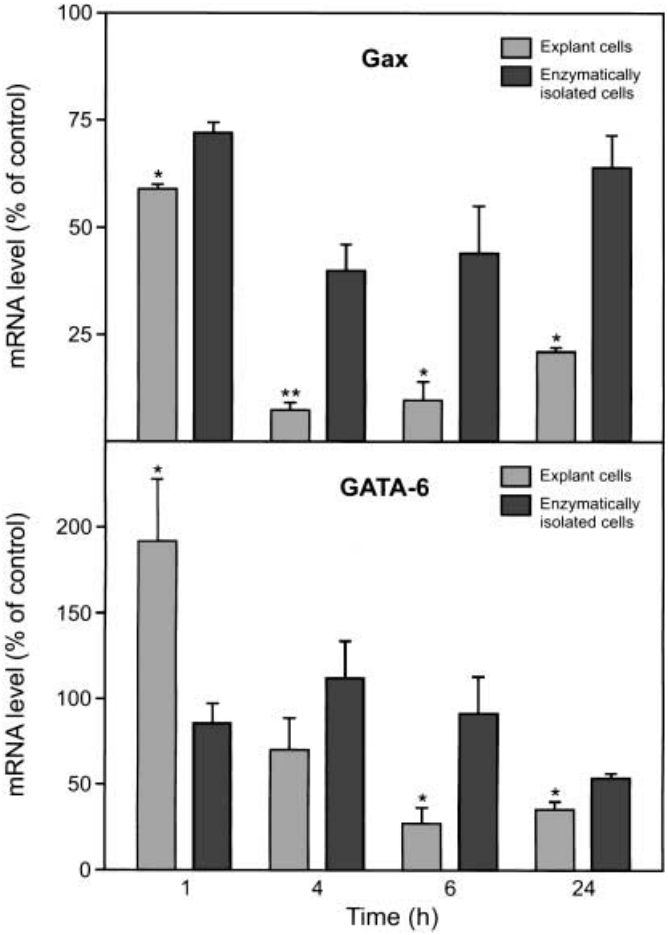

Fig. 2. Expression of the transcription factors Gax (*upper panel*) and GATA-6 (*lower panel*) by VSMC upon serum stimulation. Data are shown as percent of the mRNA quantity found in control cells maintained under serum-free conditions. Duplicate or triplicate values from four independent experiments were normalized to GAPDH mRNA and expressed as mean ± SEM. Statistically significant differences at a given time point between explant-derived and enzymatically released cells are denoted by *asterisks* (* $p < 0.05$; ** $p < 0.01$).

other side, was not influenced by serum stimulation. The occurrence of smoothelin paralleled essentially that of myosin SM2 with the exception of a faint appearance of smoothelin in regions of high cell density of explant-derived SMC maintained in the presence of serum.

After stimulation of VSMC with 10% FBS, total RNA was isolated at different time points and analysed by real-time PCR in parallel with identical total RNA quantities from non-stimulated cells. Values were normalized to GAPDH mRNA. Expression of GAPDH did not change significantly under the two different culture conditions. In three separate experiments with explant cells 127 ± 10 arbitrary units (mean ± SD) of GAPDH mRNA were found in stimulated and 112 ± 16 units in non-stimulated cells. Thus, the comparisons made in the subsequent parts of the Results section refer to similar quantities of GAPDH mRNA.

Statistically significant differences were observed when the time course of the down-regulation of Gax mRNA was compared in explant and primary cells upon serum stimulation. Within 4 h explant-derived cells responded to serum stimulation by down-regulating Gax by a factor of about 10 (Fig. 2, upper panel). In contrast, Gax became down-regulated more slowly and to a smaller extent in primary cells, although it was nevertheless statistically significant in comparison to non-stimulated cells. Statistically significant differences were also observed for changes in the expression of GATA-6. Generally, serum application led to smaller changes of GATA-6 mRNA levels when compared with Gax. After an initial up-regulation, GATA-6 mRNA declined rapidly by 75% in explant cells, whereas the initial up-regulation did not occur in primary cells, and the final reduction was only about 50% (Fig. 2, lower panel). In contrast to the serum-mediated alterations in the expression of Gax and GATA-6, the mRNA content of Oct-1 in serum-stimulated VSMC did not deviate from the value in unstimulated cells by more than ± 10% and was not statistically significant.

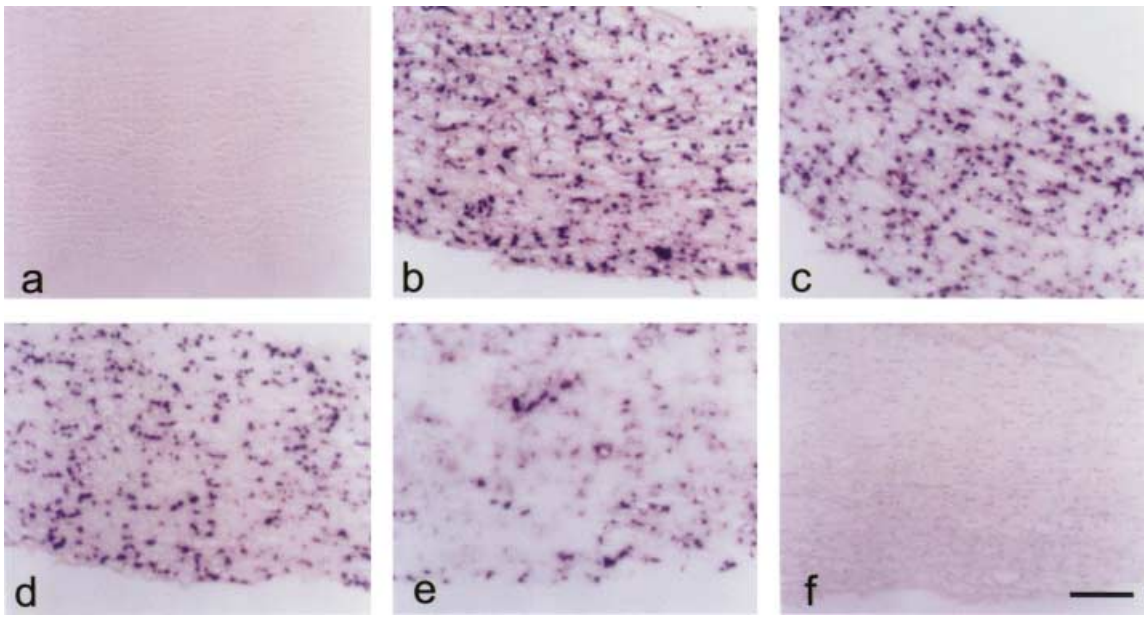

Fig. 3. Expression of Gax in media explants maintained in culture in the presence of 10% FBS. **a.** Sense probe without staining for nuclei with hematoxylin. **b – e.** Antisense probe in combination with nuclear staining. **f.** Nuclear staining only. Incubations were for 0 h (**a, b**), 2 h (**c**), 6 h (**d**) and 24 h (**e, f**), respectively. Bar, 200 μm.

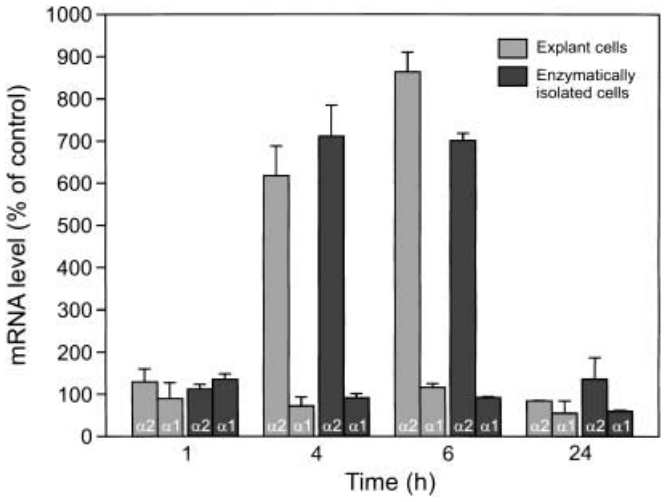

Fig. 4. Expression of the integrin chains α1 and α2 by VSMC upon serum stimulation. Further details are as described in the legend of Figure 2.

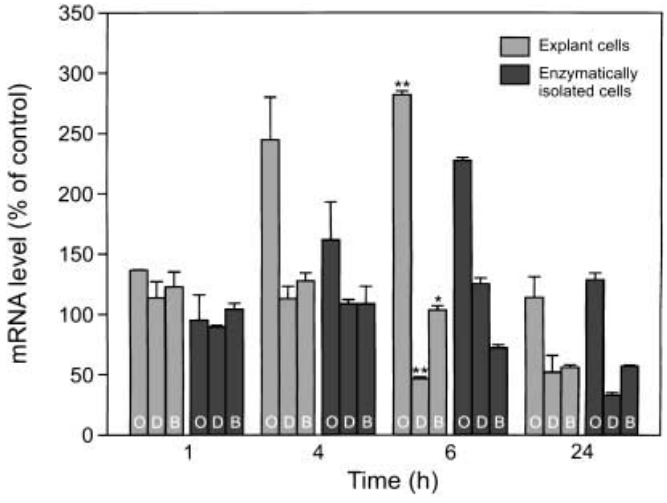

Fig. 5. Expression of osteopontin (O), decorin (D) and biglycan (B) by VSMC upon serum stimulation. Data are expressed as described in the legend of Figure 2.

In-situ hybridization of explant tissue confirms serum-mediated down-regulation of Gax

To support the cell culture finding of serum-mediated down-regulation of Gax a time-dependent series of in-situ hybridization studies of aortic explants, maintained in serum-containing medium, was performed. Figure 3 clearly indicates the dramatic loss of Gax expression with time, while the apparent expression of GAPDH remained constant (not shown), indicating the maintenance of tissue integrity. In the absence of serum, however, the GAPDH mRNA levels dropped, preventing the analysis of a control experiment in which the explants were kept under serum-free conditions.

Strong induction of integrin α2 upon serum stimulation

As the integrins α1β1 and α2β1 are reciprocally regulated in migrating VSMC (Skinner et al., 1994), the two α chains were investigated. Integrin α2 mRNA showed a very rapid but transient up-regulation by a factor of 5 to 10 after serum stimulation (Fig. 4). Both the extent and the time-course of integrin α2 induction were similar in primary and explant cells. In contrast to integrin α2, integrin α1 mRNA levels were slightly down-regulated.

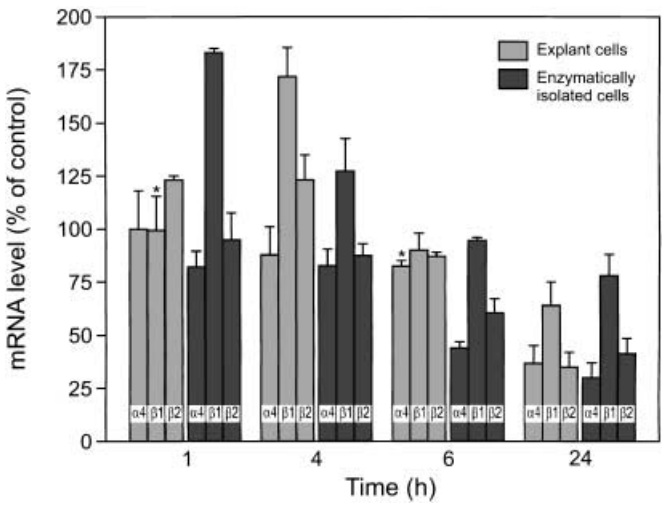

Fig. 6. Expression of laminin chains α4, β1 and β2 by VSMC upon serum stimulation. Data are expressed as described in the legend of Figure 2.

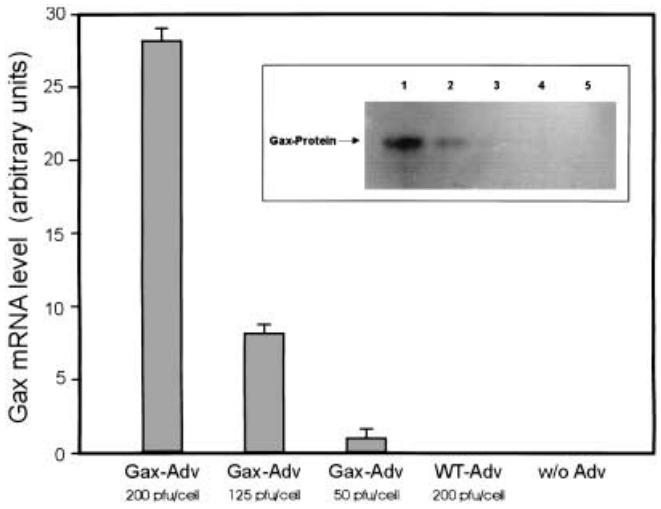

Fig. 7. Dose dependence of Gax mRNA and protein expression after infection with Gax-Adv. Explant VSMC were transfected with the virus doses indicated, and analysed after 48 h. The endogenously synthesized Gax mRNA amounts for 0.03 relative units. The Gax protein of 34 kDa was identified in cell extracts after polyacrylamide gel electrophoresis in the presence of SDS followed by Western blotting and immunological detection of Gax by enhanced chemiluminescence.

Opposite effects of serum on the expression of osteopontin and decorin

As typical components of the extracellular matrix, the expression of α1 (I) collagen, osteopontin and of the proteoglycans decorin and biglycan was studied. Serum stimulation caused a transient increase in osteopontin mRNA which occurred earlier in explant than in primary cells (Fig. 5). Decorin mRNA became reduced by 50% within 6 h (explant cells) and 24 h (primary cells), respectively, while there was a slight down-regulation of biglycan in both cell types. Expression of collagen α1 (I) mRNA changed similarly to that of biglycan (result not shown).

Up-regulation of the laminin β1 by serum stimulation

Since a basement membrane surrounds all individual VSMC and since during atherogenesis the abundance in the vessel wall of laminin 10 (α5β1γ1) increases at the expense of laminin 11 (α5β2γ1) (J. Rauterberg, unpublished observation), serum-mediated changes in the expression of several laminin polypeptide chains were studied (Fig. 6). Serum application resulted in the expected increase in the expression of the laminin β1 chain and a decrease of the β2 chain. However, this effect was observed in a statistically significant manner at earlier time-points in primary than in explant cells. Unexpectedly, primary cells showed also a more rapid decline in the expression of the α4 laminin chain which is a component of laminins 8 (α4β1γ1) and 9 (α4β2γ1). Immunohistochemically, the α4 laminin chain is mainly restricted to the endothelium in normal arterial tissue but appears around VSMC in advanced arteriosclerotic plaques (J. Rauterberg, unpublished result). The laminin γ1 chain, which is present in all laminins, except laminins 5 and 12, did not show any significant changes in mRNA expression in primary and explant cells (result not shown).

Virally induced Gax expression influences selectively integrin α2 expression

The data shown in Figure 2 clearly indicated that the expression of Gax is more sensitive towards serum stimulation than the expression of GATA-6, and it was subsequently demonstrated that the expression of several extracellular matrix-related genes is influenced by serum. To get further insight into the role of Gax on the serum-mediated phenotypic transition of VSMC, an adenovirus harbouring porcine Gax cDNA was constructed and used to infect quiescent cells. The data presented in Figure 7 demonstrate that upon infection Gax became up-regulated dose-dependently both on the mRNA as well as on the protein level. There was, however, no linear relationship between the applied dose and the effect on the expression of the transfected cDNA.

In further experiments explant-derived VSMC were infected with 125 pfu/cell of either the Gax or of the control adenovirus and stimulated 24 h later by serum addition for up to 24 h. Serum addition leads to a down-regulation of Gax mRNA which may not only be the result of a change of transcriptional activity but also of altered mRNA stability. The latter possibility may explain why there was only a moderate response towards virally induced Gax expression 6 h after serum stimulation (Fig. 8). Among the other genes investigated, also the ones not included in Figure 8 for the sake of clarity, exclusively the α2 integrin chain exhibited a significant difference of its expression level between control virus- and Gax virus-treated VSMC, respectively, when compared with the non-stimulated control. According to the data shown in Figure 4, serum stimulation correlates with a down-regulation of Gax and an increased expression of the α2 integrin gene. Thus, we proposed that in case of a regulatory role of Gax on integrin α2, treatment with the Gax virus should abolish the serum-mediated down-regulation of Gax and, hence, the up-regulation of the integrin α2 expression should either not take place at all or should reach only a modest level after serum stimulation. Indeed, 6 h after challenging the cells with serum, control virus-treated cells had a much higher integrin α2 mRNA content than Gax virus-treated VSMC, although also in the latter case a small induction of the integrin chain was

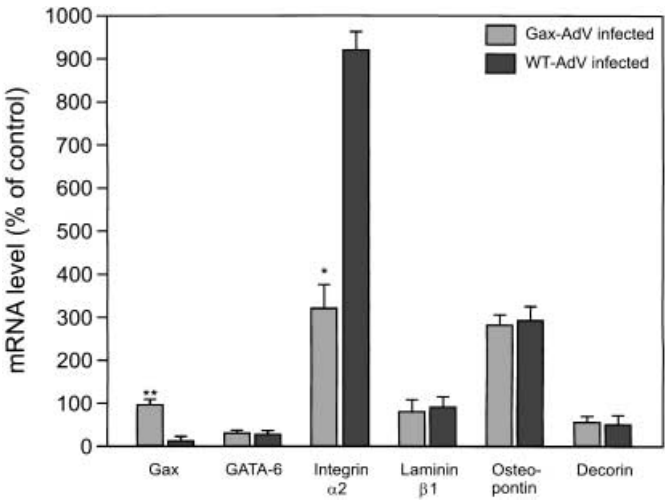

Fig. 8. Influence of virally expressed Gax on gene expression of explant VSMC. Cells were treated with 125 pfu/cell of Gax-Adv or control virus. Twenty four h after transfection VSMC were either stimulated with 10% FBS or were kept without serum addition. Six h later, cells were harvested for semiquantitative RT-PCR of the mRNAs indicated.

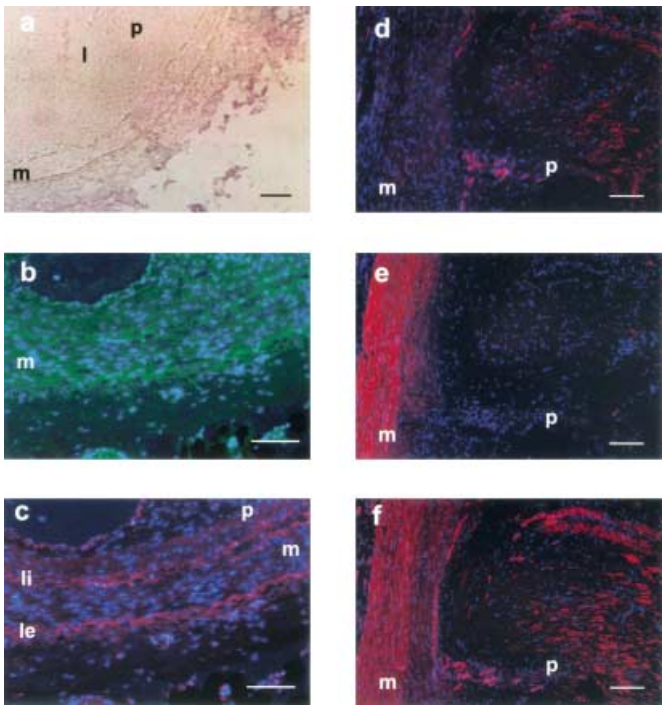

Fig. 9. In-situ hybridization for Gax (**a**), and immunohistochemical stainings for the integrin α1 (**b**) and α2 subunits (**c**) and for the laminin β1 (**d**), β2 (**e**) and γ1 (**f**) chains. Note that the elastic laminas stain non-specifically. l, lumen, le, lamina elastica externa, li, lamina elastica interna, m, media; p, plaque; bar, 100 μm.

observed (Fig. 8). A quantitatively very similar effect was seen already 4 h after serum stimulation, while there was no such difference after 24 h of incubation (data not shown).

Differences in the expression of extracellular matrix-related genes are mirrored by immunohistochemical analysis of atherosclerotic arteries

Since changes of the phenotype similar to the ones occurring in serum-induced VSMC are expected to be found during atherosclerotic plaque formation, we studied the expression of Gax by in-situ hybridization and of integrin and laminin chains by immunohistochemistry in atherosclerotic human coronary arteries. Gax expression was below the limit of detection in atherotic plaques. In contrast to our expectation, however, there was also only very low Gax expression in the medial layer of the vessel although the media appeared morphologically normal. In contrast, the adventitia yielded rather strong positive signals (Fig. 9a).

In accordance with our in vitro data it is clearly demonstrated in Figure 9b and c that the integrin α2 chain is strongly expressed in the atherosclerotic plaque whereas the integrin α1 chain is additionally present in normal media and adventitia. As a further example of the usefulness of the expression studies on cultured VSMC, immunohistochemical stainings of the laminin β1, β2 and γ1 chains are shown in Figure 9d–f. While the laminin β2 chain, being a component of laminins 9 and 11, is confined to the media, the laminin β1 chain which is present in laminins 8 and 10 is expressed exclusively in the plaque. In contrast, the laminin γ1 chain, which is a common component of all of the above mentioned laminin isoforms, is present throughout all layers of the arterial wall.

Discussion

In order to mimic the conversion of VSMC from a contractile to a synthetic phenotype, which is a hallmark of atherogenesis, VSMC were growth arrested first and subsequently challenged with FBS to provoke the phenotypic transition via serum-mediated changes of the expression of transcription factors (Nishida et al., 2002). As expected, immunohistochemistry showed that resting and serum-stimulated VSMC differed in their patterns of myosin isoforms, validating this model for studying the expression of other genes of interest involved in phenotypic transition.

The first aim of this work was to test in the model of cultured VSMC whether Gax, GATA-6 and Oct-1 are key factors responsible for switching between the contractile and synthetic phenotypes. Oct-1 did not appear as a necessary transcription factor for the conversion of the contractile into the synthetic phenotype since its expression remained unaffected by serum addition, although its expression had been considered to correlate positively with growth responsiveness of VSMC (Weiser et al., 1997). Nevertheless, Oct-1 may respond to an altered extracellular matrix produced by VSMC of the synthetic phenotype. In contrast to the expression of Oct-1, both Gax and GATA-6 were down-regulated in response to serum addition. The expression of Gax was more profoundly altered than that of GATA-6, and it could be shown that the serum-mediated down-regulation of Gax expression occurred also in tissue explants where the cells are surrounded by an intact three-dimensional extracellular matrix. We focussed, therefore, our study on the role of Gax.

Serum addition was associated with an induction of the laminin β1 chain, the α2 integrin subunit and osteopontin, whereas the laminin α4 chain and decorin were down-regulated. Surprisingly, however, it appeared from the virus infection experiments that only the expression of the α2 integrin subunit was under the direct influence of Gax. It had been shown before that the respective β chains of the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are specifically down-regulated by Gax (Witzenbichler et al., 1999), suggesting that Gax is a key regulatory factor for the expression of several integrin isoforms. In the opposite situation, the down-regulation of Gax during atherogenesis should be accompanied by a stimulation of VSMC migration. Similarly, the observed increase in the expression of $\alpha_2\beta_1$ integrin, as suggested by the data presented in this study, should enhance the migratory capacity of VSMC by collagenase (MMP-1) induction (Skinner et al., 1994; Langholz et al., 1995). Thus, in spite of the restricted direct influence of Gax on the modulation of the expression of matrix-related genes, our data support the important role of Gax during atherogenesis. In addition, an indirect effect of Gax mediated via other regulatory factors can be envisaged, as it had been shown, for example, that GATA-6, the second transcription factor which was shown in our study to respond towards serum application, is involved in the control of the thyroid transcription factor-1 (Shaw-White et al., 1999).

A second aim of our study was to compare the gene regulatory response of primary VSMC, which represent the entire spectrum of cells in the aortic media, with explant-derived VSMC characterized by high migratory and proliferative activities in their culture history. It was not intended to raise VSMC subcultures of monoclonal origin to avoid lengthy cell culture procedures in the presence of FBS. It was nevertheless anticipated that the hitherto described differences in the morphological and migratory behaviour of VSMC could be mirrored also by differences in the mRNA expression profiles of selected transcription factors and matrix proteins. Although primary and explant cells generally reacted similarly towards the proliferative stimuli of serum, remarkable quantitative and kinetic differences were indeed noted. The negative regulators of VSMC proliferation, Gax and GATA-6, were more strongly down-regulated in explant than in primary cells. In addition, GATA-6 showed an immediate early rise in explant-derived cells as previously reported in rat but not human VSMC (Suzuki et al., 1996). It is therefore likely that explant-derived VSMC represent a subpopulation of cells especially well suited to proliferate and migrate into the intima during atherogenesis.

Another notable difference between explant and primary VSMC concerned the time course of the expression changes of laminin isoforms and decorin. Explant cells responded to serum more rapidly with a decline of decorin mRNA but less rapidly with a decrease of the mRNAs for the α4 and β2 laminin chains. These differences occurred in spite of the observation that explant and primary cells contained similar quantities of these messages prior to serum stimulation. As the regulation of the expression of the different laminin isoforms is presently not understood, the latter finding awaits further studies for explanation. The more rapid down-regulation of decorin may be interpreted in the context that migrating VSMC are characterized by an activation of extracellular signal-regulated protein kinase 1,2 (Hauck et al., 2000), which results in a down-regulation of decorin (Laine et al., 2000). Down-regulation of type I collagen expression during serum stimulation of explant-derived VSMC has been described before (Kindy et al., 1988).

There is an interesting link between the expression of Gax, GATA-6 and decorin. The inhibition of cell proliferation by Gax and GATA-6 is mediated by the cyclin-dependent kinase inhibitor $p21^{Cip1/Waf1}$ (Smith et al., 1997; Perlman et al., 1998; Witzenbichler et al., 1999). Decorin-induced growth suppression is also associated with an up-regulation of $p21^{Cip1/Waf1}$ (DeLuca et al., 1996).

The third aim of this investigation was to analyse selected matrix genes the expression of which had either not yet been studied before or/and seemed to be of relevance for phenotype transition. A complex set of matrix genes is already known to change their expression during atherogenesis and restenosis (Tai et al., 2000). Regarding the present study, it is noteworthy that contractile porcine VSMC express osteopontin since hitherto this glycoprotein had been found only in atherosclerotic lesions of porcine aorta (Srivatsa et al., 1997). The observation that VSMC migration on type I collagen is supported by α2β1, but not by α1β1 integrins (Skinner et al., 1994) is reflected by the present finding that the α2 integrin chain responds rapidly to serum. The proposed repression of laminins 9 (α4β2γ1) and 11 (α5β2γ1) in VSMC of synthetic phenotype highlights the need for detailed functional investigations on basement membrane components surrounding VSMC.

As mentioned in the Results section, lack of appropriate immunological tools prevented the confirmation on the protein level of serum-mediated expression changes in porcine material. Immunohistochemical studies of human atherosclerotic coronary arteries were performed instead which were complemented by in-situ hybridization for Gax. Expression of Gax could not be observed in atherosclerotic plaques, but there was also only a very low Gax mRNA content in the morphologically unaffected medial layer of the artery. It has not yet been studied, however, how Gax is regulated in the different layers of the vascular tissue during the decade-long development of arteriosclerosis, and it may well be that Gax exerts its most important role during the initial phases of atherogenesis. In case of α1 and α2 integrin subunits as well as in case of laminin β1, β2 and γ1 chains, however, the changes observed in serum-stimulated porcine VSMC were mirrored by the results of immunochemical stainings of atherosclerotic plaques in the normal media of human coronary arteries. This may serve as an indication of the in vivo relevance of the expression data described in this study. Nevertheless, confirmation by using porcine-specific immunochemical reagents is urgently required.

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References

Becker, T. C., Noel, R. J., Coats, W. S. Gómez-Foix, A. M., Alam, T., Gerard, R. D., Newgard, C. B. (1994): Use of recombinant adenovirus for metabolic engineering of mammalian cells. Methods Cell Biol. **43**, 161 – 189.

Campbell, G. R., Campbell, J. H., Manderson, J. A., Horrigan, S., Rennick R. E. (1988): Arterial smooth muscle. A multifunctional mesenchymal cell. Arch. Pathol. Lab. Med. **112**, 977 – 986.

Campbell, J. H., Tachas, G., Black, M. J., Cockerill, G., Campbell, G. R. (1991): Molecular biology of vascular hypertrophy. Basic Res. Cardiol. **86** (Suppl. 1), 3 – 11.

DeLuca, A., Santra, M., Baldi, A., Giordano, A., Iozzo R. V. (1996): Decorin-induced growth suppression is associated with up-regulation of $p21^{Cip1/Waf1}$, an inhibitor of cyclin-dependent kinases. J. Biol. Chem. **271**, 18961 – 18965.

Dzau, V. J., Braun-Dullaeus, R. C., Sedding, D. G. (2002): Vascular proliferation and atherosclerosis: New perspectives and therapeutic strategies. Nature Med. **8**, 1249 – 1256.

Giachelli, C. M., Schwartz, S. M., Liaw, L. (1995): Molecular and cellular biology of osteopontin. Trends Cardiovasc. Med. **5**, 88 – 95.

Glukhova, M., Koteliansky, V., Fondacci, C., Marotte F., Rappaport, L. (1993): Laminin variants and integrin laminin receptors in developing and adult human smooth muscle. Dev. Biol. **157**, 437 – 447.

Gluzman, Y., Reichl, H., Solnik, D. (1982): Helper-free adenovirus type-5 vectors. In: Y. Gluzman (ed.): Eucaryotic Viral Vectors. Cold Spring Harbor Laboratory Press, New York, pp. 187 – 192.

Gorski, D. H., LePage, D. F., Patel, C. V., Copeland, N. G., Jenkins, N. A., Walsh, K. (1993): Molecular cloning of a diverged homeobox gene that is rapidly down-regulated during the G0/G1 transition in vascular smooth muscle cells. Mol. Cell. Biol. **13**, 3722 – 3733.

Gorski, D. H., Walsh, K. (1995): Mitogen-responsive nuclear factors that mediate growth control signals in vascular myocytes. Cardiovasc. Res. **30**, 585 – 592.

Gove, C., Walmsley, M., Nijjar, S., Bertwistle, D., Guille, M., Partington, G., Bomford, A., Patient, R. (1997): Over-expression of GATA-6 in *Xenopus* embryos blocks differentiation of heart precursors. EMBO J. **16**, 355 – 368.

Hauck, C. R., Hsia, D. A., Schlaepfer D. D. (2000): Focal adhesion kinase facilitates platelet-derived growth factor-BB-stimulated ERK2 activation required for chemotaxis migration of vascular smooth muscle cells. J. Biol. Chem. **275**, 41092 – 41099.

Holtkötter, O., Nieswandt, B., Smyth, N., Müller, W., Hafner, M., Schulte, V. Krieg, T., Eckes, B. (2002): Integrin α_2-deficient mice develop normally, are fertile, but display partially defective platelet interaction with collagen. J. Biol. Chem. **277**, 10789 – 10794.

Jiang, Y., Tarzami, S., Burch, J. B., Evans, T. (1998): Common role for each of the cGATA-4/5/6 genes in the regulation of cardiac morphogenesis. Dev. Genet. **22**, 263 – 277.

Kindy, M. S., Chang, C. J., Sonenshein, G. E. (1988): Serum deprivation of vascular smooth muscle cells enhances collagen gene expression. J. Biol. Chem. **263**, 11426 – 11430.

Kinsella, M. G., Tsoi, C. K., Jarveläinen, H. T., Wight, T. N. (1997): Selective expression and processing of biglycan during migration of bovine aortic endothelial cells. J. Biol. Chem. **272**, 318 – 325.

Kortesmaa, J., Yurchenco, P., Tryggvason, K. (2000): Recombinant laminin-8 ($\alpha_4\beta_1\gamma_1$). Production, purification and interactions with integrins. J. Biol. Chem. **275**, 14853 – 14859.

Laine, P., Reunanen, N., Ravanti, L., Foschi, M., Santra, M., Iozzo, R. V., Kähäri V. M. (2000): Activation of extracellular signal-regulated protein kinase 1,2 results in down-regulation of decorin expression in fibroblasts. Biochem. J. **349**, 19 – 25.

Lakin, N. D., Palmer, R., Lillycrop, K. A., Howard, M. K., Burke, L. C., Thomas, N. S., Latchman, D. S. (1995): Down regulation of the octamer binding protein Oct-1 during growth arrest and differentiation of a neuronal cell line. Brain Res. Mol. Brain Res. **28**, 47 – 54.

Langholz, O., Rockel, D., Mauch, C., Kozlowska, E., Bank, I., Krieg, T., Eckes, B. (1995): Collagen and collagenase expression in three-dimensional collagen lattices are differentially regulated by $\alpha1\beta1$ and $\alpha2\beta1$ integrins. J. Cell Biol. **131**, 1903 – 1915.

Laverriere, A. C., MacNeill, C., Mueller, C., Poelmann, R. E., Burch, J. B., Evans, T. (1994): GATA-4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut. J. Biol. Chem. **269**, 23177 – 23184.

LePage, D. F., Altomare, D. A., Testa, J. R., Walsh, K. (1994): Molecular cloning and localization of the human GAX gene to 7p21. Genomics **24**, 535 – 540.

Maillard, L., Van Belle, E., Smith, R. C., Le Roux, A., Denefle, P., Steg, G,. Barry, J. J., Branellec, D., Isner, J. M., Walsh K. (1997): Percutaneous delivery of the gax gene inhibits vessel stenosis in a rabbit model of balloon angioplasty. Cardiovasc. Res. **35**, 536 – 546.

Mano, T., Luo, Z., Malendowicz, S. L., Evans, T., Walsh K. (1999): Reversal of GATA-6 downregulation promotes smooth muscle differentiation and inhibits intimal hyperplasia in balloon-injured rat carotid artery. Circulation Res. **84**, 647 – 654.

McGrory, J., Bautista, D., Graham, F. A. (1988): A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. Virology **163**, 614 – 617.

Morrisey, E. E., Ip, H. S., Lu, M. M., Parmacek, M. S. (1996): GATA-6: a zinc finger transcription factor that is expressed in multiple cell lineages derived from lateral mesoderm. Dev. Biol. **177**, 309 – 322.

Murry, C. E., Gipaya, C. T., Bartosek, T., Benditt, E. P., Schwartz, S. M. (1997): Monoclonality of smooth muscle cells in human atherosclerosis. Am. J. Pathol. **151**, 697 – 705.

Nishida, W., Nakamura, M., Mori, S., Takahashi, M., Ohkawa, Y., Tadokoro, S., Yoshida, K., Hiwada, K., Hayashi, K., Sobue, K. (2002): A triad of serum response factor and the GATA and NK families governs the transcription of smooth and cardiac muscle genes. J. Biol. Chem. **277**, 7308 – 7317.

Perlman, H., Suzuki, E., Simonson, M., Smith, R. C., Walsh, K. (1998): GATA-6 induces $p21^{Cip1}$ expression and G_1 cell cycle arrest. J. Biol. Chem. **273**, 13713 – 13718.

Riessen, R., Isner, J. M., Blessing, E., Loushin, C., Nikol, S., Wight, T. N. (1994): Regional differences in the distribution of the proteoglycans biglycan and decorin in the extracellular matrix of atherosclerotic and restenotic human coronary arteries. Am. J. Pathol. **144**, 962 – 974.

Ross, R. (1993): The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature **362**, 801 – 809.

Schaefer, L., Raslik, I., Gröne, H.-J., Schönherr, E., Macakova, U., Ugorcakova, J., Budny, S., Schaefer, R. M., Kresse, H. (2001): Small proteoglycans in human diabetic nephropathy: discrepancy between glomerular expression and protein accumulation of decorin, biglycan, lumican and fibromodulin. FASEB J. **15**, 559 – 661.

Schaefer, L., Macakova, K., Raslik, I., Micegova, M., Gröne, H.-J., Schönherr, E., Robenek, H., Echtermeyer, F. G., Grässel, S., Bruckner, P., Schaefer, R. M. Iozzo, R. V., Kresse, H. (2002): Absence of decorin adversely influences tubulointerstitial fibrosis of the obstructed kidney by enhanced apoptosis and increased inflammatory reaction. Am. J. Pathol. **160**, 1181 – 1191.

Shaw-White, J. R., Bruno, M. D., Whitsett, J. A. (1999): GATA-6 activates transcription of thyroid transcription factor-1. J. Biol. Chem. **274**, 2658 – 2664.

Skinner, M. P., Raines, E. W., Ross, R. (1994): Dynamic expression of $\alpha1\beta1$ and $\alpha2\beta1$ integrin receptors by human vascular smooth muscle cells. $\alpha2\beta1$ integrin is required for chemotaxis across type I collagen-coated membranes. Am. J. Pathol. **145**, 1070 – 1081.

Smith, R. C., Branellec, D., Gorski, D. H., Guo, K., Perlman, H., Dedieu, J. F., Pastore, C., Mahfoudi, A., Denefle, P., Isnerm J. M., Walsh, K. (1997): $p21^{CIP1}$-mediated inhibition of cell proliferation by over-expression of the gax homeodomain gene. Genes Dev. **11**, 1674 – 1689.

Srivatsa, S. S., Fitzpatrick, L. A., Tsao, P. W., Reilly, T. M., Holmes, D. R., Jr., Schwartz, R. S., Mousa, S. A. (1997): Selective $\alpha_v\beta_3$ integrin blockade potently limits neointimal hyperplasia and lumen stenosis following deep coronary arterial stent injury: evidence for the functional importance of integrin $\alpha_v\beta_3$ and osteopontin expression during neointima formation. Cardiovasc. Res. **36**, 408 – 428.

Sturm, R. A., Das, G., Herr, W. (1988): The ubiquitous octamer-binding protein Oct-1 contains a POU domain with a homeo box subdomain. Genes Dev. **2**, 1582 – 1599.

Suzuki, E., Evans, T., Lowry, J., Truong, L., Bell, D. W., Testa, J. R., Walsh, K. (1996): The human GATA-6 gene: structure, chromosomal location, and regulation of expression by tissue-specific and mitogen-responsive signals. Genomics **38**, 283 – 290.

Tai, J. T., Brooks, E. E., Liang, S., Somogyi, R., Rosete, J. D., Lawn, R. M., Shiffman, D. (2000): Determination of temporal expression patterns for multiple genes in the rat carotid artery injury model. Arterioscler. Thromb. Vasc. Biol. **20**, 2184 – 2191.

Tamura, S., Wang, X. H., Maeda, M., Futai, M. (1993): Gastric DNA-binding proteins recognize upstream sequence motifs of parietal cell-specific genes. Proc. Natl. Acad. Sci. USA **90**, 10876 – 10880.

Thie, M., Schlumberger, W., Semich, R., Rauterberg, J., Robenek, H. (1991): Aortic smooth muscle cells in collagen lattice culture: effects on ultrastructure, proliferation and collagen synthesis. Eur. J. Cell Biol. **55**, 295 – 304.

Thyberg, J. (1996): Differentiated properties and proliferation of arterial smooth muscle cells in culture. Int. Rev. Cytol. **169**, 183 – 265.

Tunggal, P., Smyth, N., Paulsson, M., Ott, M. C. (2000): Laminins: structure and genetic regulation. Micros. Res. Techn. **51**, 214 – 227.

Vischer, P., Buddecke, E. (1985): Alteration of glycosyltransferase activities during proliferation of cultivated arterial endothelial cells and smooth muscle cells. Exp. Cell Res. **158**, 15 – 28.

Weiser, M. C., Grieshaber, N. A., Schwartz, P. E., Majack, R. A. (1997): Perlecan regulates Oct-1 gene expression in vascular smooth muscle cells. Mol. Biol. Cell **8**, 999 – 1011.

Weitkamp, B., Cullen, P., Plenz, G., Robenek, H., Rauterberg, J. (1999): Human macrophages synthesize type VIII collagen in vitro and in the atherosclerotic plaque. FASEB J. **13**, 1445 – 1457.

Winer, J., Jung, C. K. S., Shakel, I., Williams, P. M. (1999): Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal. Biochem. **270**, 41 – 49.

Witzenbichler, B., Kureishi, Y., Luo, Z., Le Roux, A., Branellec, D., Walsh, K. (1999): Regulation of smooth muscle cell migration and integrin expression by the Gax transcription factor. J. Clin. Invest. **104**, 1469 – 1480.

Yamakawa, T., Bai, H., Masuda, J., Sawa, Y., Shirakura, R., Ogata, J., Matsuda, H. (2000): Differential expression of proteoglycans biglycan and decorin during neointima formation after stent implantation in normal and atherosclerotic rabbit aortas. Atherosclerosis. **152**, 287 – 297.